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Interaction of Neuronal Calcium Sensor-1 (NCS-1) with Phosphatidylinositol 4-Kinase β Stimulates Lipid Kinase Activity and Affects Membrane Trafficking in COS-7 Cells*

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Phosphatidylinositol 4-kinases (PI4K) catalyze the first step in the synthesis of phosphatidylinositol 4,5-bisphosphate, an important lipid regulator of several cellular functions. Here we show that the Ca^{2+}-binding protein, neuronal calcium sensor-1 (NCS-1), can physically associate with the type III PI4Kβ with functional consequences affecting the kinase. Recombinant PI4Kβ, but not its glutathione *S*-transferase-fused form, showed enhanced PI kinase activity when incubated with recombinant NCS-1, but only if the latter was myristoylated. Similarly, *in vitro* translated NCS-1, but not its myristoylation-defective mutant, was found associated with recombinant- or *in vitro* translated PI4Kβ in PI4Kβ-immunoprecipitates. When expressed in COS-7 cells, PI4Kβ and NCS-1 formed a complex that could be immunoprecipitated with antibodies against either proteins, and PI 4-kinase activity was present in anti-NCS-1 immunoprecipitates. Expressed NCS-1-YFP showed co-localization with endogenous PI4Kβ primarily in the Golgi, but it was also present in the walls of numerous large perinuclear vesicles. Co-expression of a catalytically inactive PI4Kβ inhibited the development of this vesicular phenotype. Transfection of PI4Kβ and NCS-1 had no effect on basal PIP synthesis in permeabilized COS-7 cells, but it increased the wortmannin-sensitive [^{32}P]phosphate incorporation into phosphatidylinositol 4-phosphate during Ca^{2+}-induced phospholipase C activation. These results together indicate that NCS-1 is able to interact with PI4Kβ also in mammalian cells and may play a role in the regulation of this enzyme in specific cellular compartments affecting vesicular trafficking.

Inositol lipid kinases are increasingly recognized as regulators of membrane remodeling events whether in Golgi-related transport, endocytosis, or exocytosis (1). These enzymes catalyze the formation of specific inositol phospholipids, which, in turn, contributes to the membrane recruitment and stabilization of molecular complexes via interaction of inositides with protein motifs present in several regulatory proteins. Phosphatidylinositol (PI)[1] 4-kinases (PI4Ks) are the enzymes that catalyze the formation of PI(4)P, the main precursor of several other polyphosphoinositides with important regulatory functions. PI 4-kinase activities have been characterized some 15–20 years ago and have been classified as type II and type III enzymes, based on their catalytic properties (2). Molecular identification of these proteins has been relatively slow, but two forms of type III PI4Ks have been cloned from various species. These enzymes, a larger (~200 kDa) α, and a smaller (~100 kDa) β form, are mammalian homologues of the yeast STT4 and PIK1 gene products, respectively, and are greatly conserved in all eukaryotes, including plants (3–5). Type II PI4K(s) have been purified from several tissues, but their molecular identity has only recently been elucidated (6, 7).

Although both Stt4 and Pik1 are *bona fide* PI4Ks, they appear to serve nonredundant functions in yeast. Stt4 has been shown to participate in cell wall synthesis, while Pik1 is involved in Golgi-related trafficking (8–10). In mammalian cells, PI4Kβ is primarily localized to the Golgi (11), where it has been reported to be regulated by Arf proteins (12). The function(s) and exact site(s) of PI4Kα action(s) still await clarification. Both of these enzymes can be inhibited in mammalian cells by micromolar concentrations of the PI 3-kinase inhibitor, wortmannin, and at these higher concentrations, wortmannin completely inhibits the resynthesis of PI(4)P and PI(4,5)P_2 in agonist-stimulated cells. This observation led to the assumption that hormone-sensitive inositide pools are also synthesized by type III PI 4-kinase(s) (13). Both the α and β forms are present in high concentrations in the brain and may participate in the membrane recycling events that are associated with synaptic transmission, since phosphoinositides have also been implicated in this process (14).

Recently, it has been reported that the yeast homologue of the Ca^{2+}-dependent regulatory protein, NCS-1, is able to stimulate PI 4-kinase activity of yeast homogenates apparently through interaction with the Pik1 protein (15). NCS-1 was first identified in *Drosophila* (where it was named frequenin) as an important determinant of synaptic plasticity and a regulator of synaptic development (16). Homologues of NCS-1 have been found in *Xenopus* (17) as well as in avian (18) and mammalian tissues (19), and together with recoverin/neurocalcin they form a group of small Ca^{2+}-binding proteins distinct from calmodu-

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[1] The abbreviations used are: PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-monophosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI4K, phosphatidylinositol 4-kinase; NCS-1, neuronal calcium sensor-1; GST, gluthathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GFP, green fluorescent protein.

lin (20). The present study was undertaken to investigate whether mammalian NCS-1 is able to interact and regulate PI4Kβ in mammalian cells. Our results indicate that the two proteins can directly interact *in vitro* and that NCS-1 exerts a moderate stimulatory effect on the lipid kinase activity of PI4Kβ. The present data also show that myristoylation of NCS-1 is critical for its ability to interact with PI4Kβ and that expressed NCS-1-YFP co-localizes with PI4Kβ in the Golgi and induces the appearance of multiple perinuclear vacuoles. Analysis of the synthesis of endogenous PI(4)P in permeabilized COS-7 cells indicate that although overexpression of PI4Kβ and NCS-1 has detectable effects on PI(4)P synthesis during Ca^{2+}-activated PI(4,5)P_2 hydrolysis, these effects are relatively minor compared with the amounts of the expressed proteins. These data suggest that PI4Kβ interacts with NCS-1, but this complex probably requires additional factor(s) to access the endogenous substrate PI and, hence, regulate PI(4)P synthesis in a specific cellular compartment.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal anti-NCS-1 antibody 44162 (21) was used in these studies. The polyclonal anti-PI4Kβ antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Protein A-agrose was purchased from Calbiochem and the TNT T7 Quick Coupled Trascription/Traslation System from Promega (Madison, WI). [γ-^{32}P]ATP (3000–6000 Ci/mmol) and [^{35}S]methionine were purchased from PerkinElmer Life Sciences. ATP, adenosine, and WT were obtained from Sigma. Phosphatidylinositol was purchased from Fluka (Ronkonkoma, NY). The *N*-myristoylated p22 protein and the polyclonal antibody raised against it were kindly provided by Dr. Margarida. Barroso (University of Virginia, Charlottesville, VA) (22). All other reagents were of analytical or high pressure liquid chromatography grade.

DNA Constructs—The rat NCS-1 cDNA was originally amplified from rat brain cDNA using the polymerase chain reaction and its sequence was found identical to that deposited in the GenBank™ (accession number L27421). A Kozak consensus sequence was added by polymerase chain reaction to the NCS-1 cDNA, and the amplified cDNA was subcloned into the *Bam*HI/*Xho*I sites of pcDNA3 (Invitrogen). The myristoylation mutant of NCS-1 was generated by converting the Gly at position 2 to Ala. This mutant abolishes myristoylation of the protein, as shown by the lack of incorporation of [^{3}H]myristic acid. The NCS-1-YFP plasmid was constructed by cloning the NCS-1 cDNA in frame in front of the yellow fluorescent protein in the pEYFP-N1 plasmid. The sequence of all constructs was verified by automatic dideoxy sequencing. The mammalian expression plasmid encoding bovine PI4Kβ has been described earlier (23).

In Vitro Translation—One microgram of supercoiled DNA plasmid was transcribed *in vitro* and then translated in the presence of [^{35}S]methionine with the TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The reaction products were analyzed by SDS-PAGE followed by autoradiography or were used further in the protein association assays.

Production of Recombinant Proteins and in Vitro Protein-Protein Interaction Assay—Expression and purification of the PI4Kβ-glutathione *S*-transferase (GST) fusion protein was performed as described previously (24). Fusion proteins were cleaved with the PreScission protease and analyzed on a SDS-polyacrylamide gel for integrity and to quantitate the amount of each batch of the purified protein. The *N*-myristoylated NCS-1 was either produced in *Escherichia coli* that also expressed *N*-myristoyltransferase (25), or the protein was expressed in SF9 cells using the Bac to Bac system (Life Technologies, Inc.) according to the manufacturer's instructions. In both cases the protein was purified with hydrophobic interaction chromatography (25).

PreScission-cleaved PI4Kβ (~2–3 μg) or 5 μl of the [^{35}S]methionine-labeled *in vitro* translated PI4Kβ was incubated with 5 μl of [^{35}S]methionine-labeled translation product of the *N*-myristoylated or mutant NCS-1 in 200 μl of binding buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, and 5 μg of ethidium bromide). The reaction was allowed to proceed for 1 h at 4 °C with gentle rocking after which anti-PI4Kβ antibody and protein A-agarose beads were added for incubation at 4 °C overnight. Agarose beads were subjected to five rounds of washing with 1 ml of binding buffer each time. The beads were resuspended in 30 μl of SDS-PAGE sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE, and the gel was treated with EN^{3}HANCE (DuPont) before drying and autoradiography.

Immunocytochemistry—For immunostaining, COS-7 cells were grown on coverslipes and fixed in 4% formaldehyde in 0.1 M PBS (pH = 7.4) for 30 min at room temperature. After three washes with 0.1 M PBS (5 min each), fixed cells were incubated in blocking solution (0.1% bovine serum albumin and 0.06% of Triton X-100 in PBS) for 1 h to decrease the nonspecific binding of the antibodies and to improve the penetration of the antibodies through membranes. This blocking solution was also used for diluting the primary and secondary antibodies. After 1 h, the blocking solution was changed for the primary antibody solution (1:2500), and the cells were incubated at 4 °C overnight. After three washes, cells were incubated with a fluorescence-labeled secondary antibody (1:1000, goat anti-rabbit IgG-Alexa 568, Molecular Probes, Eugene, OR) for 1 h at room temperature. This was followed by a last washing step (3 × 5 min, in PBS), then the cells were rinsed with distilled water, air-dried, and mounted on glass slides using Cytoseal 60 mounting medium (Stephens Scientific). Cells were then analyzed by confocal microscopy.

Immunoprecipitation and Western Blot Analysis—COS-7 cells were cultured on 10-cm culture dishes and transfected with the LipofectAMINE 2000 Reagent (Life Technologies, Inc.) as described previously (24). Transfected COS-7 cells were rinsed three times with ice-cold PBS, and cell lysates were prepared by the addition 1.3 ml of ice-cold lysis buffer (20 mM Hepes (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, 2 mM EDTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin) to each 10-cm plate and scarping into 15-ml tubes. After sonication samples were transferred to Eppendorf tubes, centrifuged at 20,000 × *g* for 20 min, and assayed for protein concentration by the BCA Protein Assay (Pierce). To an aliquot of the lysates containing equal amounts of protein, 1 μg of anti-PI4Kβ antibody or 2 μg of anti-NCS-1 antibody (44162) was added, and samples were incubated at 4 °C overnight. Immunoprecipitated proteins were collected by protein A-Sepharose. After washing five times with 0.6 ml of lysis buffer, samples were subjected to SDS-PAGE analysis. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore), and Western blotting was performed with the respective antibodies. Alternatively, immunoprecipitates on protein A beads were analyzed for PI kinase activity.

Assay of PI 4-Kinase—The activity of PI 4-kinase was measured as incorporation of radioactivity from [γ-^{32}P]ATP into organic solvent-extractable material as described previously (13). The standard reaction mixture for PI 4-kinase (50 μl final volume) contained 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$,1 mM EGTA, 1 mM PI, 0.4% Triton X-100, 0.5 mg/ml bovine serum albumin (lipid kinase buffer), 100 μM [γ-^{32}P]ATP, and the enzyme. When the Ca^{2+} sensitivity of the kinase was examined, the PI kinase medium was titrated with Ca^{2+} in the presence of Fura-2 free acid in a fluorescence spectrophotometer. The desired Ca^{2+} concentration was then adjusted by adding the appropriate amount of Ca^{2+} read from this titration curve. All assay components except [γ-^{32}P]ATP were preincubated with or without inhibitors for 10 min at room temperature. Reactions were started by the addition of [γ-^{32}P]ATP and terminated after 10 min by the addition of 3 ml of $CHCl_3$/CH_3OH/37% HCl (200:100:0.75 (v/v/v)). The organic solvent phase was separated from [γ-^{32}P]ATP as described elsewhere (26), and after evaporation, its activity was determined in a liquid scintillation counter.

Permeabilized Cell Studies—COS-7 cells were seeded on 12-well plates (40,000 cells/well) and cultured for 2 days before transfection with the LipofectAMINE 2000 reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were washed with PBS and were incubated in 400 μl of permeabilization medium containing 100 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.4), 2 mM EDTA, 0.05% bovine serum albumin, 15 μg/ml digitonin, 0.3 mM ATP, 12 μCi/ml [γ-^{32}P]ATP, and the various stimuli. Incubations were carried out at 37 °C for 10 min, and reactions were terminated with perchloric acid (5% final). Inositol lipids were extracted and separated by TLC as described previously (27), and their radioactivity was quantitated by a PhosphorImager.

RESULTS

Stimulation of Recombinant PI4Kβ Lipid Kinase Activity by Recombinant NCS-1—We have previously shown that bacterially expressed bovine PI4Kβ is functional with properties that are indistinguishable from those of the purified bovine enzyme

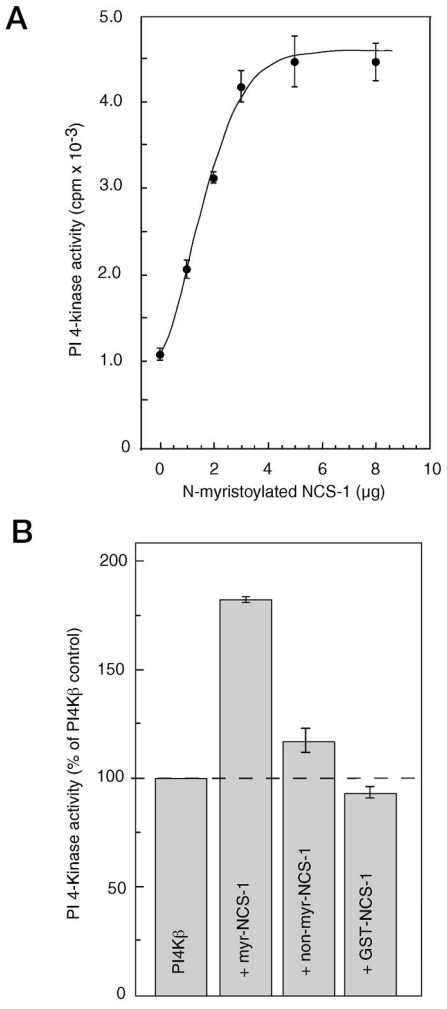

FIG. 1. **Stimulation of the PI 4-kinase activity of recombinant PI4Kβ by recombinant NCS-1.** Recombinant PI4Kβ was produced in bacteria as a GST fusion protein and cleaved with the PreScission protease. Myristoylated NCS-1 was either produced in bacteria also expressing *N*-myristoyltransferase or in the Sf9 cells. Purified proteins were incubated together for 15 min, and the activity of the kinase was measured with PI and [γ-^{32}P]ATP as substrates. *A* shows a representative result of one of three observations, performed in triplicates, while *B* shows the summary of three to four experiments (mean ± S.E.).

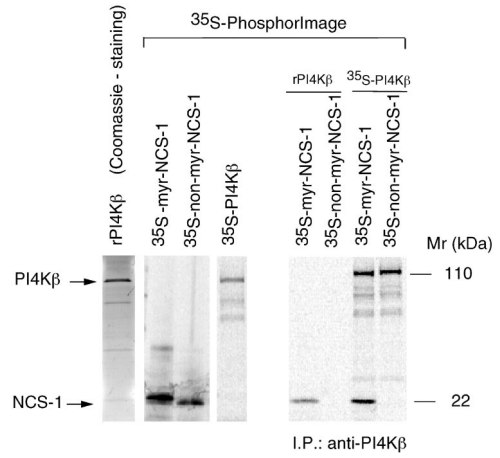

FIG. 2. **Association of NCS-1, but not its myristoylation-defective mutant, with PI4Kβ in a cell-free system.** [^{35}S]Methionine-labeled PI4Kβ, NCS-1, or its myristoylation-defective mutant, G2A-NCS-1, were produced by *in vitro* translation for binding studies. The labeled NCS-1 products were incubated either with recombinant PI4Kβ (*rPI4Kβ*) or with the *in vitro* transcribed, labeled PI4Kβ for 1 h at 4 °C, before immunoprecipitation with an anti-PI4Kβ antibody overnight. Immunoprecipitated samples were subjected to SDS-PAGE, followed by analysis on a PhosphorImager. The *left panel* shows the components that were used for the binding reaction, and the *right panel* shows what was found in the PI4Kβ immunoprecipitates. Note the lack of association of the nonmyristoylated NCS-1, which migrates slightly faster than NCS-1 in the SDS gels. This experiment was repeated with identical results.

(24). To investigate whether NCS-1 is able to alter the activity of PI4Kβ, we used the recombinant protein either in its GST-fused or native form after cleavage of the GST moiety. NCS-1 was also expressed in bacteria either as a nonmyristoylated protein or its myristoylated form that was produced in bacteria also expressing *N*-myristoyltransferase (25). A GST fusion protein of NCS-1 was also produced. As shown in Fig. 1*A*, myristoylated NCS-1 (*myr-NCS-1*) was able to stimulate *in vitro* PI 4-kinase activity of recombinant PI4Kβ in a dose-dependent manner. The extent of stimulation varied between NCS-1 preparations, and it averaged around a 2-fold increase in the course of these studies. Nonmyristoylated NCS-1 was much less effective, although it evoked a minor but reproducible effect on PI4Kβ (Fig. 1*B*). GST-fused NCS-1 was without effect on PI 4-kinase activity, consistent with the need for myristoylation. Similarly, no significant effect of NCS-1 was observed when the GST-fused form of PI4Kβ was used (not shown), indicating that an interaction may require N-terminal sequences of the kinase that could be obscured by the GST molecule.

Since NCS-1 is a Ca^{2+}-binding protein, next we examined whether Ca^{2+} can affect PI4Kβ activity in the presence of myristoylated NCS-1. We have previously examined the Ca^{2+} sensitivity of partially purified type III PI4K from bovine adrenal (which mostly contains the β form of type III PI 4-kinase) and found no effect of the cation, even in the presence of bovine brain calmodulin (26). Changing Ca^{2+} concentration in the micromolar range further stimulated PI4Kβ in the presence of myristoylated NCS-1, but NCS-1 stimulated PI4Kβ even without added Ca^{2+}. Stimulation by Ca^{2+} was not more than 2-fold in average and did not show the dose-response relationship that would be consistent with the Ca^{2+}-induced conformational change described recently (28) (data not shown).

To investigate whether the effects of *N*-myristoyl NCS-1 on PI4Kβ activity is simply due to the hydrophobic myristoyl group, we examined the effect of recombinant *N*-myristoylated p22, another small Ca^{2+}-binding protein (22), on the activity of recombinant PI4Kβ. However, we found that this protein had no stimulatory effect regardless of the Ca^{2+} concentration used in these experiments, and we could not demonstrate an association between these two proteins (data not shown).

N-Myristoylated NCS-1 Associates with PI4Kβ—Next we examined whether the interaction of the two proteins is strong enough to be analyzed by immunoprecipitation. For this, we used an *in vitro* translated NCS-1 or its myristoylation-defective mutant, G2A-NCS-1. The [^{35}S]methionine-labeled translated proteins were incubated either in the presence of recombinant PI4Kβ or with an *in vitro* translated [^{35}S]methionine-labeled PI4Kβ. After incubation for 1 h, proteins were subjected to immunoprecipitation with anti-PI4Kβ antibody and analyzed for the presence of ^{35}S-labeled NCS-1. As shown in Fig. 2, only the myristoylated NCS-1 was found in the PI4Kβ-immunoprecipitates despite a comparable amount of labeled nonmyristoylated NCS-1 present in the binding reaction. Also, nonmyristoylated NCS-1 (which migrated slightly faster than myristoylated NCS-1 on SDS gels) that was present in small amounts, even in the *in vitro* translation product of wild-type NCS-1, was never observed in the anti PI4Kβ immunoprecipitates. These results also confirmed that the two proteins can physically interact, and myristoylation of NCS-1 is required for their efficient association.

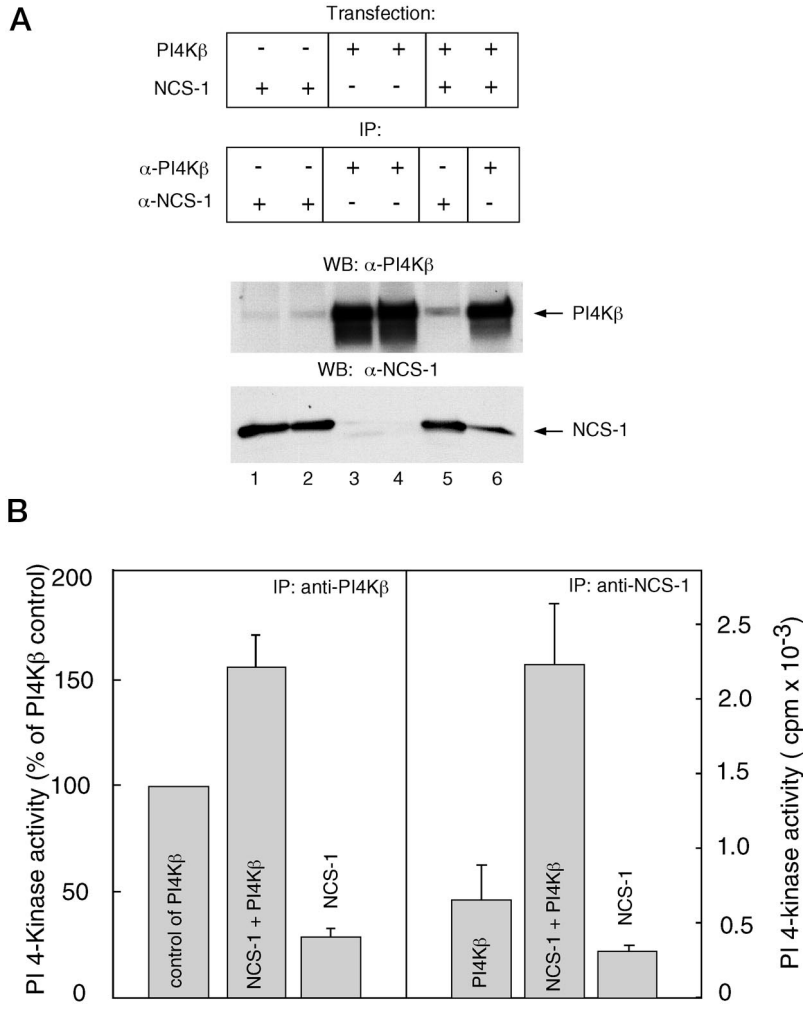

FIG. 3. **Association of expressed NCS-1 with PI4Kβ in COS-7 cells.** COS-7 cells were transfected with cDNA plasmids encoding NCS-1 or PI4Kβ or with their combination as indicated in *A*. Two days after transfection, cells were lysed and immunoprecipitated (*IP*) either with an anti-PI4Kβ or an anti-NCS-1 antibody. Immunoprecipitated proteins were subjected either to Western blot (*WB*) analysis using both anti-PI4Kβ and anti-NCS-1 antibodies (*A*) or to a PI 4-kinase assay (*B*). Results shown are mean ± S.E. ($n = 3$; *B*, *left*) and mean ± range of two independent experiments (*right*).

Interaction of NCS-1 and PI4Kβ in COS-7 Cells—While these studies indicated that myristoylated NCS-1 and PI4Kβ can associate *in vitro*, we wanted to extend these observations to mammalian cells and examine whether such physical association between the two proteins can be demonstrated within mammalian cells. For this reason, we transfected PI4Kβ, NCS-1, or their combination into COS-7 cells. After transfection and cell lysis, either NCS-1 or PI4Kβ was immunoprecipitated, and the complex was analyzed for PI 4-kinase activity or was subjected to Western analysis to show the presence of the respective proteins in the complex. As shown in Fig. 3*A* association of the two proteins was demonstrable by Western analysis of the immunoprecipitates. It is also shown that without transfection, no endogenous NCS-1 (or a related protein) was detected in COS-7 cells associated with the expressed PI4Kβ. In contrast, a small amount of endogenous PI4Kβ was found associated with overexpressed and immunoprecipitated NCS-1. It is also clear from these data that only a fraction of the expressed proteins can be found in association.

When the PI 4-kinase activity of the precipitates was analyzed, a 50% increase of PI 4-kinase activity in the anti-PI4Kβ immunoprecipitates was observed in cells where PI4Kβ and NCS-1 were co-expressed, compared with cells expressing PI4Kβ alone (the green fluorescent protein was expressed along with PI4Kβ in place of NCS-1 in these experiments as control). When the anti-NCS-1 antibody was used for immunoprecipitation, a small PI 4-kinase activity was observed in the lysates where NCS-1 or PI4Kβ was expressed alone, but a significant increase was observed when the two proteins were expressed together (Fig. 3*B*). The PI 4-kinase activity was always inhibited by 1 μM wortmannin throughout these experiments (not shown), indicating that the activity is associated with the type III β enzyme.

Effects of Overexpression of PI4Kβ and NCS-1 on the Phosphorylation of Endogenous PI in Permeabilized Cells—Since the PI4K activity assays described above used the soluble PI substrate in Triton X-100 micelles, they would not reveal any regulation affecting the access of the enzyme to its lipid substrate in the actual membrane environment within the cells. Therefore, we performed experiments in COS-7 cells in which the two proteins were overexpressed and used permeabilization with digitonin (15 μg/ml) and [γ-^{32}P]ATP to determine the rate of PI(4)P synthesis from the endogenous substrate. As shown in Fig. 4, no significant increase in the ^{32}P incorporation into PI(4)P was observed under basal conditions in cells expressing PI4Kβ alone or with NCS-1. This was despite the large amounts of overexpressed active PI4Kβ protein found in the cells (see Fig. 3), indicating that the presence of excess enzyme has little if any detectable impact on PI phosphorylation. To determine whether the effect of PI4Kβ overexpression becomes more apparent during an increased "PI turnover," we examined PI(4)P synthesis during Ca^{2+} stimulation of phospholipase C activity. In control cells (expressing GFP only), elevated Ca^{2+} (~100 μM) causes the breakdown of PI(4,5)P_2 and PI(4)P, which is reflected in the decreased labeling of both of these phospholipids. However, significanty higher [^{32}P]phosphate incorporation into PI(4)P was observed in the presence of Ca^{2+} in COS-7 cells overex-

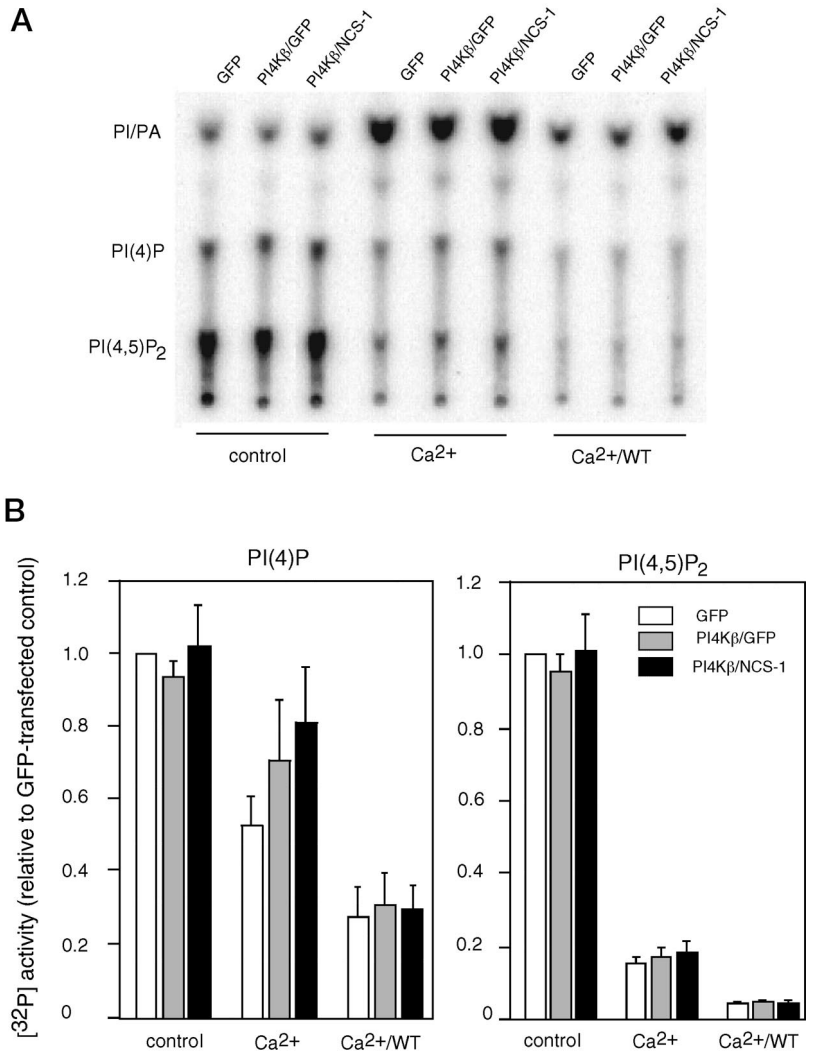

FIG. 4. **[^{32}P]Phosphate labeling of PI(4)P and PI(4,5)P_2 in permeabilized COS-7 cells expressing PI4Kβ and NCS-1.** COS-7 cells were cultured on 12-well plates and transfected with the indicated constructs for 24 h. GFP was used only to keep the amount of transfected DNA equal. After washing, cells were permeabilized with digitonin in an "intracellular" type medium containing [γ-^{32}P]ATP with or without Ca^{2+} (~100 μM) and were incubated for 10 min. Wortmannin (10 μM) was added 10 min prior to permeabilization. Reactions were terminated by adding perchloric acid, and after extraction, lipids were separated by TLC. *A* shows a TLC run from a representative experiment, and *B* shows the combined results from five experiments, each performed in duplicates. (Mean ± S.E. are shown and the values are expressed as percent of the absolute control.) The difference in the labeling of PI(4)P in the presence of Ca^{2+} between the control (GFP) and PI4Kβ/GFP- and PI4Kβ/NCS-1-transfected groups was statistically significant when the values were normalized to the Ca^{2+}-treated control PI(4)P value ($p < 0.05$, between each groups, analyzed with the Duncan test).

pressing PI4Kβ, an effect that was further enhanced by NCS-1 co-expression. This increased incorporation was completely blocked by 10 μM wortmannin (Fig. 4), consistent with the involvement of the PI4Kβ. While these effects were small, it has to be emphasized that an increased PI4Kβ activity in a specific membrane compartment may not have a large impact on the overall PI(4)P synthesis. Therefore, these observed changes are important in that the effect of PI4Kβ and NCS-1 overexpression can be detected also on endogenous substrates during a high rate of PI(4)P consumption.

Localization of NCS-1 in COS-7 Cells—To investigate the cellular distribution of overexpressed NCS-1 and PI4Kβ in live cells, we used a chimeric protein in which YFP was fused to the C terminus of NCS-1 so that the N-terminal myristoylation sequence of NCS-1 is not affected. COS-7 cells were transfected with this construct and examined by confocal microscopy. As shown in Fig. 5, *A–C*, fluorescence was present throughout the cytoplasm, as well as in the nucleus, but it was enriched in structures consistent with Golgi localization as well as in the plasma membrane. No such localization was observed with a myristoylation-defective mutant NCS-1-YFP (not shown). Importantly, a significant fraction of cells (up to 60–70%) showed multiple large vesicular structures at their perinuclear region, and NCS-1 was present in the lining of these vesicles (Fig. 5, *A–C*). To determine whether PI4Kβ was also present in the wall of these large vesicles, we performed immunostaining with a PI4Kβ antibody performed on fixed cells. These experiments showed clear co-localization of PI4Kβ in the Golgi, but no particular enrichment of the kinase was observed in the wall of the vesicular structures (Fig. 5, *D–F*). Similar results were obtained when PI4Kβ was also overexpressed except for more intense PI4Kβ staining in the cytosol (not shown). It should be noted that significantly less and smaller vesicles were observed on fixed cells than on the live cell images.

We also used a PI4Kβ construct fused at its C terminus to GFP to assess the cellular distribution of the kinase and the impact of NCS-1 expression on its localization. This construct showed mostly cytosolic distribution and a clear localization to the Golgi, but we could not see any notable change in its distribution upon co-expression of NCS-1. However, we noted that in these cells, expressing PI4Kβ-GFP, no vesicles developed in response to NCS-1 expression (data not shown). Since the GFP-tagged PI4Kβ looses its catalytic activity,[2] we further investigated whether a kinase inactive mutant of PI4Kβ (D656A) (24) could also interfere with the morphological effects caused by NCS-1-YFP expression. These experiments showed that a significantly smaller fraction of cells showed the multiple perinuclear vesicles (less than 20%) when cells were co-transfected with PI4Kβ-D656A, and even in cells that showed such vesicles their number and size were greatly reduced (Fig. 5, *G–I*). These results together indicated that the morphological

[2] X.-H. Zhao and T. Balla, unpublished observation.

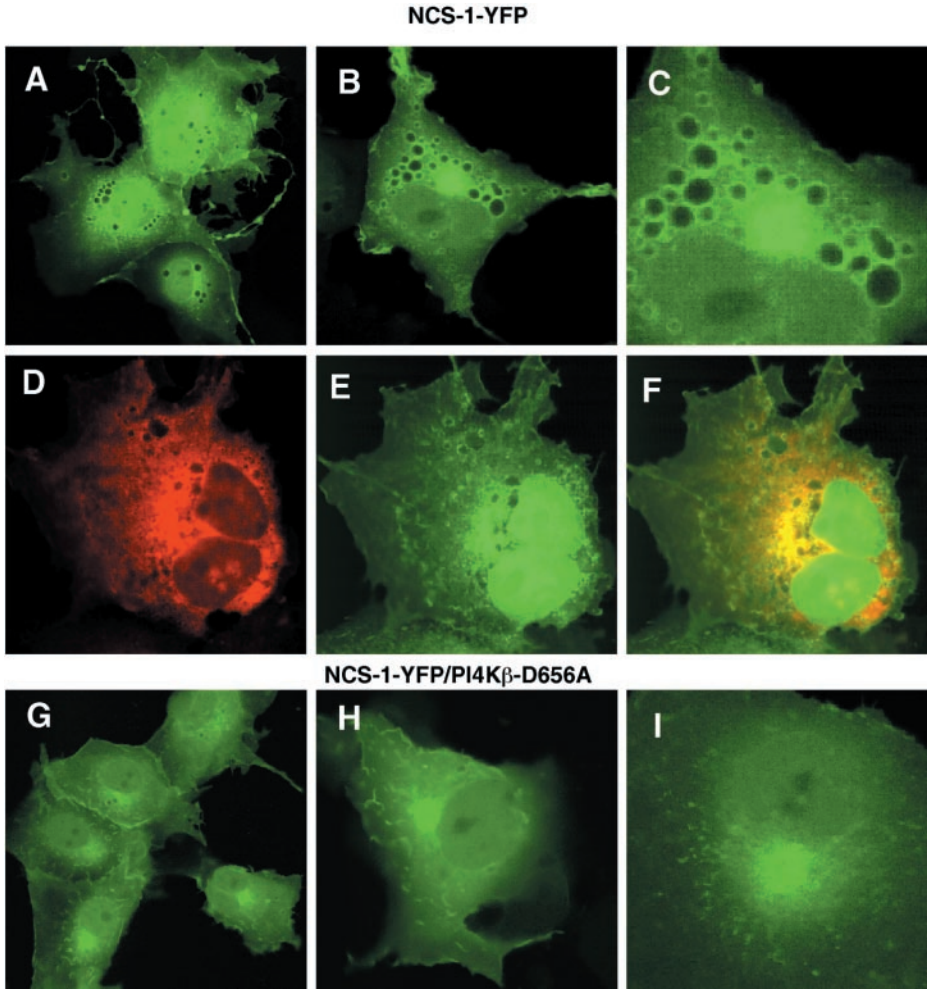

FIG. 5. **Localization of NCS-1-YFP and PI4Kβ in COS-7 cells.** COS-7 cells, grown on glass coverslips, were transfected with a cDNA plasmid encoding the NCS-1 protein fused at its C terminus to the enhanced yellow fluorescent protein (*NCS-1-YFP*). After 1 day, live cells were examined in an inverted laser confocal microscope (Bio-Rad, MR 1024). The YFP signal is most prominent over the area that corresponds to the Golgi, but is also present in the plasma membrane and the walls of perinuclear vesicles (*A–C*). For immunocytochemistry transfected cells were fixed and immunostained with a primary antibody against PI4Kβ and a secondary antibody labeled with Alexa-568 (*red*). Cells were observed with a laser confocal microscope using 488 nm and 568 nm laser lines and simultaneous detection with 525-nm and 585-nm long pass filters. Crossover of the two signals was negligible as assessed by using only one excitation each at a time. The co-localization of NCS-1-YFP (*green*) with the endogenous PI4Kβ over the Golgi is clearly visible, but no particular enrichment in PI4Kβ is seen at the plasma membrane and in the walls of vesicular structures (*D–F*). *G–I* show that co-expression of a catalytically inactive PI4Kβ greatly reduces the formation of the perinuclear vesicles in cells expressing NCS-1-YFP.

effects of NCS-1 overexpression in COS-7 cells require a catalytically active PI4Kβ.

Addition of ionomycin to increase cytosolic Ca^{2+} concentration did not cause an obvious acute change in the distribution of NCS-1-YFP. Similarly, treatment with 10 μM wortmannin was without an acute effect (up to 10 min) on the localization of the protein.

DISCUSSION

In the present study we provide evidence for the association and possible regulation of PI4Kβ activity by the Ca^{2+}-binding regulatory protein, NCS-1, in mammalian cells. This regulation has been shown previously in *Saccharomyces cerevisiae* based on both genetic and biochemical evidence (15). Our data using recombinant proteins indicate that PI4Kβ and NCS-1 can interact without any additional binding partner, although it cannot be ruled out that, in the intact cell, additional proteins or lipids may participate and modify the interaction between these two proteins. Our studies indicate that myristoylation of NCS-1 is critical for efficient interaction and stimulation of PI4Kβ, although in the yeast study a similar difference was not observed (15). However, in the same yeast study the myristoylation-defective mutant of yeast frequenin was found much less effective than wild type in suppressing a temperature-sensitive *pik1* allele (15). In the yeast, the N-terminal lipid kinase unique domain was found to be the site of NCS-1 binding to PIK1. The activity of GST-fused PI4Kβ was not affected by NCS-1 in our studies, also indicating the involvement of N-terminal sequences on PI4Kβ in the association. Although we found that Ca^{2+} can further stimulate the activity of the NCS-1-PI4Kβ complex, Ca^{2+} was not essential for the association of the two proteins, and the presence or absence of Ca^{2+} did not make a noticeable difference in our immunoprecipitation experiments. Similarly, Ca^{2+} was found not to be required for the association of the two proteins in yeast (15).

Overexpression of PI4Kβ with or without NCS-1 had little if any impact on the [^{32}P]phosphate labeling of endogenous PI(4)P in permeabilized COS-7 cells under basal condition. However, the two proteins still exerted a small but significant effect on [^{32}P]phosphate labeling of PI(4)P during Ca^{2+}-induced phospholipase C activation, confirming the functional interaction between the two proteins. These results are consistent with our observation that recombinant PI4Kβ is not able to phosphorylate the endogenous PI of red blood cell membranes,[3] indicating that a putative adapter molecule assists the kinase in its access to the membrane-bound substrate. These data, as well as those on the cellular localization of the two proteins (see below), also suggest that NCS-1 alone is not the adapter that determines the localization of the kinase and that NCS-1 stimulates the kinase only in a membrane subdomain, most likely related to the Golgi.

Our studies on the localization of the two proteins expressed in COS-7 cells are consistent with the biochemical data showing interaction of the two proteins. Both proteins are localized to the Golgi, as are their endogenous forms as shown in a recent study (29). However, NCS-1-YFP is also found in certain membranes (plasma membrane, nuclear membrane, and vacuolar

[3] T. Balla, unpublished results.

membranes) where the localization of PI4Kβ is less prominent or completely lacking. We found no indication that NCS-1 would change its localization in response to the Ca^{2+} ionophore, ionomycin. This is in agreement with recent findings on the Ca^{2+} insensitivity of myristoylated NCS-1 binding to rat brain membranes (30). The most prominent effect of the overexpression of NCS-1-YFP was the formation of large perinuclear vacuoles with NCS-1 present in their membranes in the majority of the cells expressing this protein. Although these structures did not show particular enrichment in PI4Kβ, the co-expression of a catalytically inactive mutant PI4Kβ, or its catalytically inactive GFP fusion form, was able to prevent the development of this characteristic vacuolar phenotype. These data strongly argue that PI4Kβ mediates the effects of NCS-1, leading to the development of the morphological changes. Intriguingly, in a recent report Weisz *et al*. (21) have shown that overexpression of NCS-1 caused a defect in the apical transport of influenza hemagglutinin from the trans-Golgi network, without affecting early transport steps from the Golgi in MDCK cells. All these data together are consistent with an important role of Arf-1, NCS-1, and PI4Kβ in the Golgi (12) and subsequent vesicular trafficking steps, similarly to the role of PIK1 in yeast (9).

The tissue distribution and reported effects of NCS-1 together suggest that the physiological function(s) of this protein is to regulate neuroendocrine secretion and transmitter release (31). While overexpression of NCS-1 significantly enhanced purinergic stimulation of secretion in adrenal chromaffin cells, it failed to affect Ca^{2+}-induced secretion in the same permeabilized cell preparation (30, 31). This finding indicates that despite being a Ca^{2+}-binding protein, NCS-1 may not serve at the Ca^{2+}-dependent final step of exocytosis. A connection between the function(s) of NCS-1 and PI4Kβ in the secretory process and/or presynaptic events is quite feasible in light of several studies, indicating the importance of inositides in the exocytic fusion event (32) as well as in the process of neurotransmitter release (14). Phosphoinositides, and the kinases and phosphatases that regulate their levels, are clearly emerging as critical players at many cellular processes involving membrane budding or fusion events. Therefore, while NCS-1 and PI4Kβ might act in concert in regulating Golgi-related vesicular transport steps in COS-7 cells, the association between the two proteins could affect additional membrane events in neurons or secretory cells. While the present study demonstrates that the two proteins can physically associate with functional consequences on PI4Kβ activity, it still remains to be determined in which membrane compartment they function together to control exocytosis and neurotransmitter release.

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